

# Immunological Studies on *Pityrosporum* Genus and *Malassezia furfur*

MICHIAKI TANAKA, M.D., AND SADA O IMAMURA, M.D.

Department of Dermatology, Osaka Saiseikai Nakatsu Hospital, Osaka, Japan (MT) and Department of Dermatology, Faculty of Medicine, Kyoto University, Kyoto, Japan (SI)

The antigenicity of *Malassezia furfur* from patients with tinea versicolor and 3 species of *Pityrosporum* was investigated using the antiserum against *P. orbiculare*. The Ouchterlony gel diffusion test revealed a considerable similarity between the antigenicities of *P. orbiculare* and *P. ovale*, and little similarity between *P. orbiculare* and *P. canis*. Similar results were obtained by immunofluorescence staining with the FITC-labeled *P. orbiculare* antiserum. Hyphae and round spores of *M. furfur* in the scales and biopsy specimens from the lesions in patients with tinea versicolor revealed specific fluorescence much the same as seen in cases of *P. orbiculare* or *P. ovale*. The FITC-labeled antiserum absorbed with *P. orbiculare* or *P. ovale* failed to give a positive reaction with hyphae and round spores of *M. furfur*. These findings suggest a similar antigenicity among *P. orbiculare*, *P. ovale* and *M. furfur*.

*Malassezia furfur* (*M. furfur*), the etiologic agent of tinea versicolor, is characterized by hyphae and round spores. Gordon [1,2] first isolated *Pityrosporum orbiculare* (*P. orbiculare*) from tinea versicolor scales and differentiated it from *Pityrosporum ovale* (*P. ovale*) which is often found in human dandruff. Both *P. orbiculare* and *P. ovale* are lipophilic and considered to be components of normal skin flora. Two difficult problems remained to be clarified. One concerns the relationship between *P. orbiculare* and *P. ovale*, the other between *M. furfur* and *P. orbiculare*. Some investigators pointed out the possible identity of *P. orbiculare* and *P. ovale* morphologically [3-5], physiologically [6], and immunologically [7]. The following support the view that the yeast-like cells of *P. orbiculare* and hyphae of *M. furfur* represent different phases of the same organism: the successful production of tinea versicolor on the human normal skin by *P. orbiculare* [8], application of fluorescent antibody technique on *M. furfur* in tinea versicolor scales and on cultured *P. orbiculare* [9,10], demonstration of similar fine structures of both fungi [3,11] and the induction of hyphae in cultures of *Pityrosporum* [12]. We attempted to determine the antigenic relationship among 3 species of *Pityrosporum* and *M. furfur* using immunofluorescence staining method and the Ouchterlony gel diffusion test with *P. orbiculare* antiserum.

## MATERIALS AND METHODS

### Preparation of *Pityrosporum* Antigens

Strains of *P. orbiculare*, *P. ovale* and *Pityrosporum canis* (*P. canis*), kindly provided by Dr. Soh, Kobe Municipal Central Hospital, Japan, were grown on the medium containing malt extract (Difco) 20 gm, yeast extract (Difco) 2 gm, dextrose 20 gm, peptone 20 gm, agar 20 gm and olive oil 1-2%, the volume of which was adjusted to 1000 ml by adding distilled water. After being washed several times with 99% ethanol and dried, the yeasts were crushed in a mill and shaken in 100 volumes of 1/15 M phosphate buffer solution (pH 7.4) at 4°C for 24 hr. After centrifugation at 11,400 g for 20 min, the supernatant fluids were filtered through a Millipore filter (0.22 µ) and stored as the soluble

antigen of *P. orbiculare*, *P. ovale* or *P. canis*. The protein concentration of each antigen was 0.28 mg/ml, 0.45 mg/ml and 0.88 mg/ml, respectively.

### Preparation of *P. orbiculare* Antiserum

*P. orbiculare* was suspended in saline at a concentration of 100 mg/ml and mixed with an equal volume of incomplete Freund's adjuvant. This mixture was given intramuscularly to 2 rabbits weighing 2.0 to 2.5 kg in the amount of 2.0 and 3.0 ml, respectively. One month later, the animals were given 4 courses of intravenous administration of the suspension of *P. orbiculare* at a concentration of 1.0 mg/ml. Inoculation was given on 3 successive days followed by a 4 day rest. The initial dose of 0.5 ml was injected, and the dose was increased by 0.5 ml each day until a final dose of 5.0 ml was given. On the 28th day, the animals were exsanguinated. The sera from both rabbits showed a 1:128 precipitin titer against *P. orbiculare* and such was pooled to be used for the Ouchterlony gel diffusion test and immunofluorescence staining.

### Ouchterlony Gel Diffusion Test

The anti-*P. orbiculare* rabbit serum was tested by the method of Ouchterlony [14] against 3 soluble antigen solutions prepared from *P. orbiculare*, *P. ovale* and *P. canis*.

### Conjugation of Antiserum

The crude globulin fraction of the *P. orbiculare* antiserum was purified by three precipitations with one-third saturated ammonium sulfate at 4°C. The globulin was conjugated with FITC for 6 hr by the method of Marshall, Evelaud, and Smith [13], at a dye-to-protein ratio of 1:60. To remove nonspecific staining material, the conjugate was fractionated through a DEAE cellulose column. The protein content and fluorescein-protein ratio of the conjugate were shown to be 13.7 mg/ml and 0.6, respectively.

### Preparation for Staining

Yeasts of *Pityrosporum* genus and the scales of lesions from 22 patients with tinea versicolor were put in a drop of distilled water on nonfluorescent glass slides and smeared. The biopsy specimens from the lesions in 12 patients with tinea versicolor were quickly frozen in an acetone Dry Ice mixture. Smears of yeasts or scales and frozen sections were fixed in 95% ethanol for 10 min. Smears of yeasts were stained with anti-*P. orbiculare* conjugate at 37°C for 2 hr. Smears of scales and frozen sections were stained at 37°C over night. The absorption test was carried out as follows: the anti-*P. orbiculare* conjugate was absorbed with *P. orbiculare*, *P. ovale* or *P. canis*. *Pityrosporum* genus was stained with these absorbed anti-*P. orbiculare* conjugates. A fluorescence microscope, equipped with ultraviolet light from a maximum pressure mercury lamp, was used for observation of the specimens.

## RESULTS

### Ouchterlony Gel Diffusion Test

On Ouchterlony gel diffusion, 3 bands of precipitates were visible against *P. orbiculare*, 2 against *P. ovale* and 1 against *P. canis* (Fig 1). The fusion of the bands of precipitates was recognized on the first band which was common to 3 species of *Pityrosporum* and on the second band which was common to *P. orbiculare* and *P. ovale*. *P. orbiculare* disclosed a third band which was also found indistinctly in *P. ovale*. There was no fusion of the third bands of precipitates between *P. orbiculare* and *P. ovale*. It is, therefore, concluded that soluble antigens of *P. orbiculare* and *P. ovale* contain at least 2 antigenic components in common, and those of *P. orbiculare* and *P. canis* contain at most one antigenic component in common.

Manuscript received January 31, 1979; accepted for publication May 2, 1979.

Reprint requests to: Dr. M. Tanaka, Department of Dermatology, Osaka Saiseikai Nakatsu Hospital, 10-39, 2 Chome, Shibata, Kita-ku, Osaka 530, Japan.

### Immunofluorescence Staining of Smears of *Pityrosporum* Genus

When tested with anti-*P. orbiculare* conjugate, *P. orbiculare* and *P. ovale* revealed intense apple-green fluorescence (with a 3+ reaction) especially at the margin of yeasts and there was no visible difference in intensity between both organisms (Fig 2 and 3). *P. canis* reacted weakly (with a 1+ reaction). The results obtained by absorption test were as follows: *P. orbiculare* and *P. ovale* reacted weakly with the anti-*P. orbiculare* conjugate absorbed with *P. canis* but did not react at all with that absorbed with *P. orbiculare* or *P. ovale*. There was no visible difference in the reaction between *P. orbiculare* and *P.*

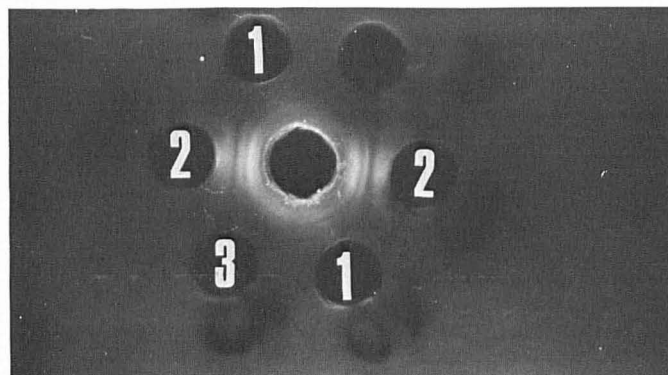
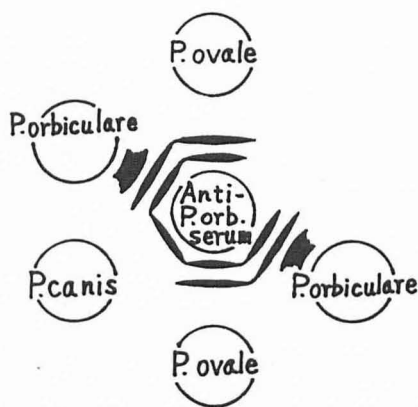


FIG 1. Ouchterlony gel diffusion test. Central well: anti-*P. orbiculare* rabbit serum. Peripheral wells: soluble antigen solutions of *P. ovale* (1), *P. orbiculare* (2) and *P. canis* (3).

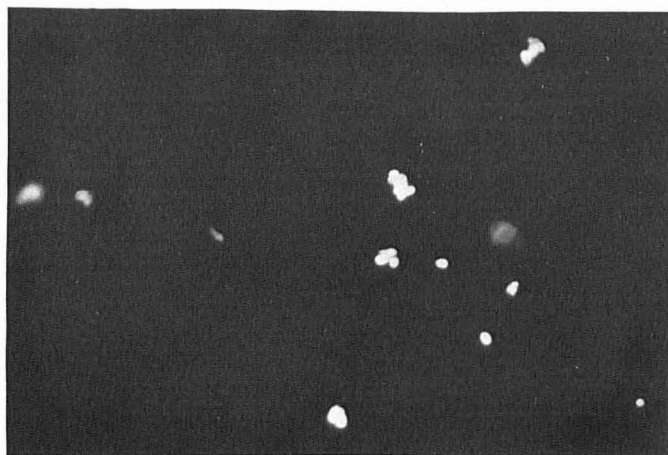


FIG 2. *P. orbiculare* in a smear stained with anti-*P. orbiculare* conjugate ( $\times 560$ ).

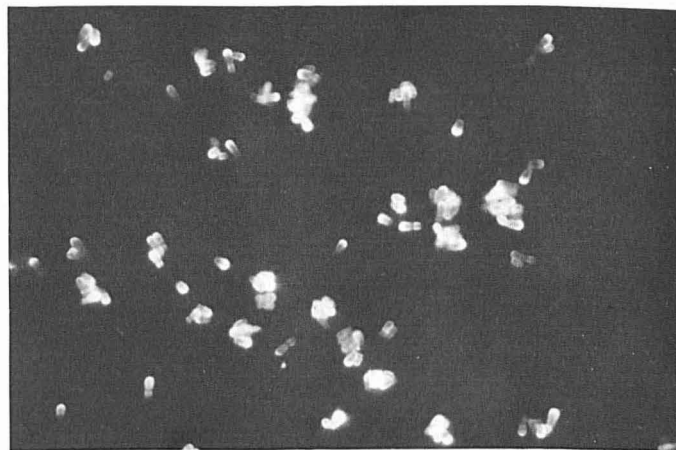


FIG 3. *P. ovale* in a smear stained with anti-*P. orbiculare* conjugate ( $\times 560$ ).

### Immunofluorescence staining and absorption test for 3 species of *Pityrosporum* in smears and for *M. furfur* in scales

Smear of	Not absorbed	Anti- <i>P. orbiculare</i> conjugate		
		Absorbed with		
		<i>P. canis</i>	<i>P. ovale</i>	<i>P. orbiculare</i>
<i>P. orbiculare</i>	3+ <sup>a</sup>	1+	—	—
<i>P. ovale</i>	3+	1+	—	—
<i>P. canis</i>	1+	—	—	—
<i>M. furfur</i>	2+	1+	—	—

<sup>a</sup> 1+: weakly positive, 2+: positive, 3+: intensely positive.

*ovale*. *P. canis* did not react with any of anti-*P. orbiculare* conjugate absorbed with *P. orbiculare*, *P. ovale* or *P. canis* (Table).

### Immunofluorescence Staining of *M. furfur* in Scales and Biopsy Specimens from Patients with Tinea Versicolor

In all scales from 22 patients with tinea versicolor, not only hyphae but also round spores in the scales gave a positive reaction (Fig 4). When anti-*P. orbiculare* conjugate was applied, there was no difference in the intensity of reaction among 22 patients (with a 2+ reaction). In the absorption test, *M. furfur* in the scales reacted weakly with the anti-*P. orbiculare* conjugate absorbed with *P. canis*, but the specific fluorescence of *M. furfur* was disappeared when the conjugate was absorbed with *P. orbiculare* or *P. ovale* (Table). In all biopsy specimens obtained from 12 patients with tinea versicolor, *M. furfur* was recognized in the stratum corneum, giving a positive reaction (with a 2+ reaction) (Fig 5). In one case *M. furfur* was found to exist in the orifice of the follicle (Fig 6). Round spores were also visible in the frozen section but such were often undistinguishable from the transversal section of hyphae which simulated to the round spores.

### DISCUSSION

There are 3 types of species of *Pityrosporum* genus. *P. orbiculare* and *P. ovale* can be isolated from human skin and are lipophilic whereas such is not the case with *P. canis*. In our study herein, both Ouchterlony's method and the direct immunofluorescence staining suggested that *P. canis* differed from the other 2, antigenically. Gordon [1] proposed the term "*P. orbiculare*" because it differed from *P. ovale* in several morphological and physiological characteristics. However, whether or not *P. orbiculare* actually differed from *P. ovale* remained to be determined mainly because *P. orbiculare* in primary cultures from tinea versicolor occasionally produced the oval

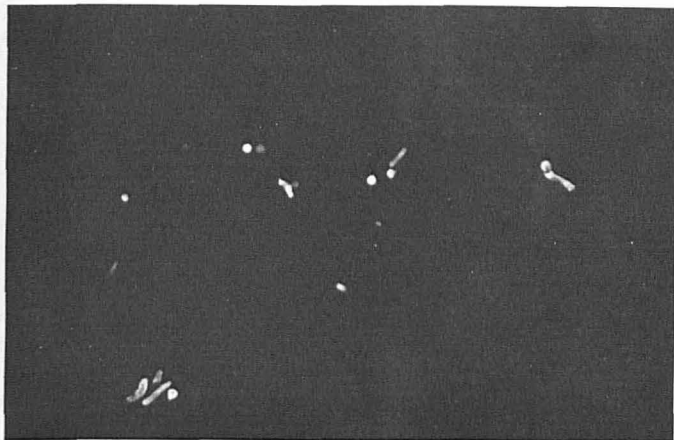


FIG 4. Hyphae and round spores of *M. furfur* in scales of lesions from patients with tinea versicolor, stained with anti-*P. orbiculare* conjugate ( $\times 560$ ).

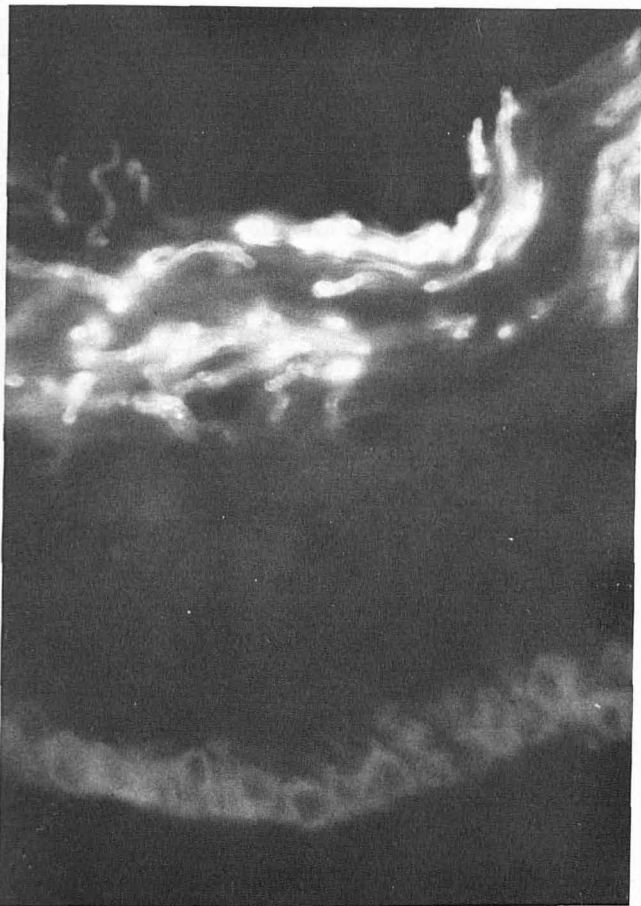


FIG 5. *M. furfur* in the stratum corneum, demonstrated by anti-*P. orbiculare* conjugate ( $\times 560$ ).

type identical with *P. ovale* morphologically and physiologically after several transfers [4,5]. Some antigenic relationship between both yeasts could be speculated upon from the study of Alexander [7] dealing with antibody titers to *P. orbiculare* and *P. ovale* using an immunofluorescent technique. It was thereafter suggested that *P. orbiculare* and *P. ovale* could be ascribed to the same species based on findings in physiological [6] and electron microscopical [3] investigations. Our results

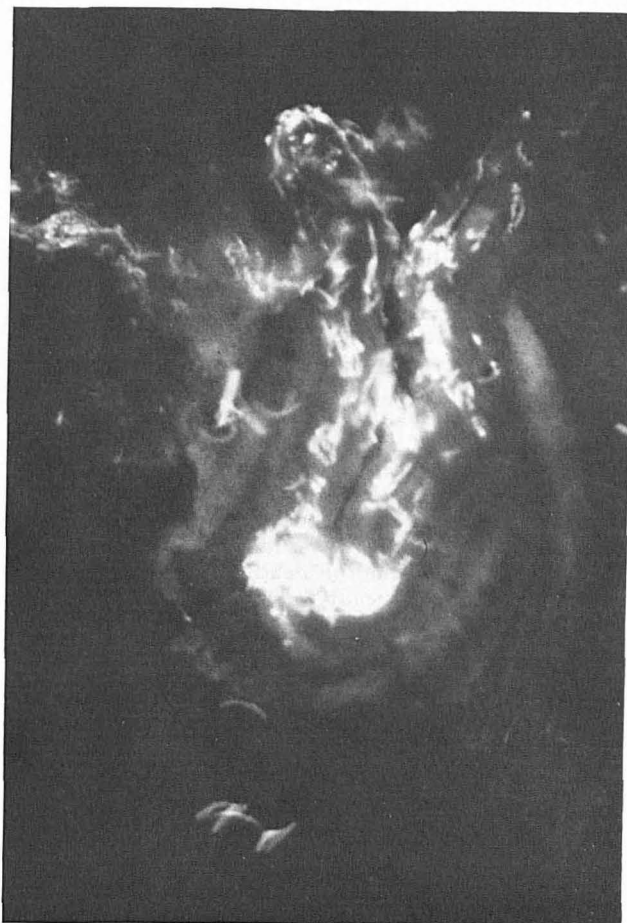


FIG 6. *M. furfur* in the orifice of a follicle as demonstrated by anti-*P. orbiculare* conjugate ( $\times 224$ ).

obtained by the method of Ouchterlony indicated that *P. orbiculare* and *P. ovale* had at least 2 common antigens, and that *P. orbiculare* had a third antigen which was also found indistinctly in *P. ovale*. It is not evident whether these third antigens are identical or not, as the third bands of precipitates of *P. orbiculare* and *P. ovale* did not fuse. Immunofluorescence staining of these 2 species with the anti-*P. orbiculare* conjugate revealed a similar intensity of the apple-green fluorescence and such was particularly evident as a ring-shape surrounding the cells. When *P. orbiculare* was stained with the anti-*P. orbiculare* conjugate absorbed with *P. ovale*, the fluorescence was practically nil though bright blue autofluorescence was present. Such result, differing from those obtained by the method of Ouchterlony, suggests the identity of third antigens of both yeasts, and the discrepancy may be attributed to the difference in sensitivities of the tests. Results obtained with the direct immunofluorescence staining, however, which detects surface antigens, cannot be directly compared with those of the Ouchterlony gel diffusion test, which probably also detects many intracellular antigens.

The possible identity of *P. orbiculare* and *M. furfur* was suggested by some investigators. Burke [8] succeeded in producing experimental infection, using tinea versicolor scales or cultured *P. orbiculare*. Keddie and Shadomy [9] reported that the antiserum immunized with *P. orbiculare* was shown to bind to *M. furfur* by the indirect fluorescent antibody procedure. Sternberg and Keddie [10] obtained the similar results by the indirect immunofluorescence staining with the serum of patient with tinea versicolor which showed the high antibody titer to *P. orbiculare*. Fundamental differences were not recognized



between these organisms, electron microscopically [3,11]. These reports suggested that *P. orbiculare* and *M. furfur* represented different phases of the same organism.

Another aspect concerns the problem of whether or not the round spore of *M. furfur* in the scales from tinea versicolor is *P. orbiculare* itself. Recently, Hayami [3] stated that both *P. orbiculare* and spores of *M. furfur* had similar fine structures. Keddie and Shadomy [9] observed the similar intensity of both hyphae and spores of *M. furfur* by the indirect fluorescent antibody staining with the antiserum immunized with *P. orbiculare*. More recently, Nazzaro-Porro et al [12] succeeded in the induction of hyphae, such being similar to those of *M. furfur* in the tissues, in cultures of *Pityrosporum* with cholesterol and cholesterol esters.

We applied the direct immunofluorescence staining with anti-*P. orbiculare* conjugate to both *Pityrosporum* genus and *M. furfur* in the lesions of patients with tinea versicolor. Both hyphae and spores of *M. furfur* presented the apple-green fluorescence, which was similar to that of *P. orbiculare* or *P. ovale*, in the biopsy specimens as well as in the scales. The fluorescence disappeared after the conjugate was absorbed with *P. orbiculare* or *P. ovale*. The results obtained in our investigation strongly suggest that the identity between *M. furfur* and *P. orbiculare* is antigenically convincing, and that the round spore of *M. furfur* may indeed be *P. orbiculare* itself.

We thank M. Ohara, Kyoto University, for pertinent advice on the manuscript.

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